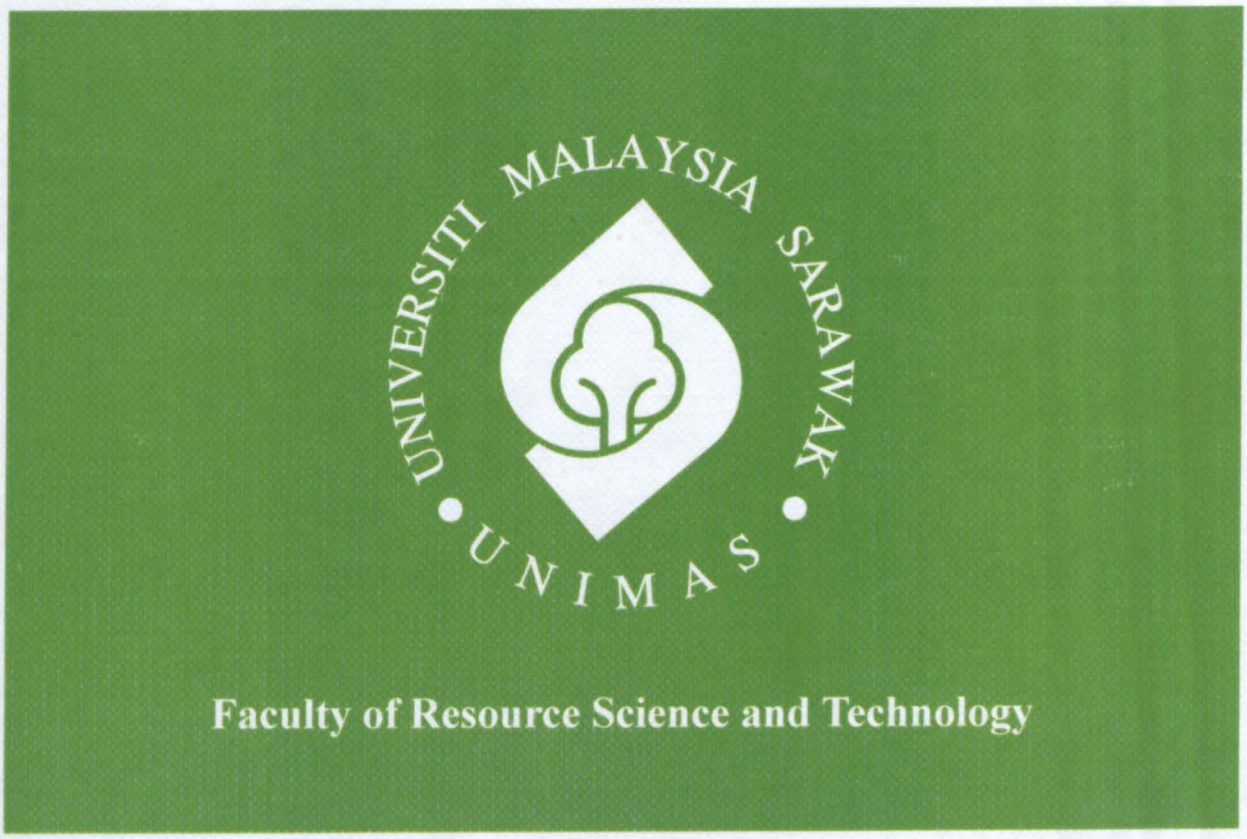

Isolation and Characterization of Methicillin-Resistant *Staphylococcus aureus* from Air Conditioning System in Faculty of Resource Science and Technology, Universiti Malaysia Sarawak

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(53489)

Bachelor of Science with Honours
(Resource Biotechnology)
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Nurul Nadia Binti Zayadi (53489)

A thesis submitted in fulfilment of the Final Year Project (STF 3013) Course

Faculty of Resource Science and Technology

UNIVERSITI MALAYSIA SARAWAK

2018

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Isolation and Characterization of Methicillin-Resistant *Staphylococcus aureus* (MRSA) from Air Conditioning System in Faculty of Resource Science and Technology, Universiti Malaysia Sarawak

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ABSTRACT

Staphylococcus aureus (*S. aureus)* is one of major human pathogen that able cause mild to serious infections. Present treatment becomes challenging to control due to the presence of methicillin-resistant *Staphylococcus aureus* (MRSA). The present study looks on the isolation and characterization of MRSA from air conditioning system in Faculty of Resources Science and Technology, UNIMAS. The swab samples were isolated from the ventilation of central air conditioner and dipped into 0.1 % peptone water. The swab samples were then carried out for serial dilution and plating on mannitol salt agar (MSA) and incubated for 24-36 hours in room temperature. Suspected colonies of *S. aureus* appeared as whitish colonies on MSA agar, and have gram-positive cocci in clusters. Only 16.67% of the total samples with dilution factor of 10^{-5} were positive for suspected *S. aureus* and were chosen to proceed for the following biochemical test. However, only 25% of the isolates were tested positive for catalase test, gelatin liquefaction test, and methyl red test, except for citrate test and urease test. All of the isolates were then carried out to assess their resistance towards four antibiotics by using disc diffusion method. All of the isolates were considered as resistant towards methicillin, penicillin, vancomycin, and cephalothin. This study is significant to increase awareness to UNIMAS staffs, lecturers, and students, by providing basic data for UNIMAS authorities on the status of MRSA from air conditioning system in UNIMAS.

Key words: Methicillin-resistant *Staphylococcus aureus* (MRSA), mannitol salt agar (MSA), disc diffusion method.

ABSTRAK

Staphylococcus aureus ialah salah satu daripada patogen yang mampu menyebabkan infeksi yang sederhana kepada yang lebih serius. Perawatan terkini semakin mencabar disebabkan kemunculan Methicillin-resistant Staphylococcus aureus (MRSA). Kajian ini bertujuan untuk megisolasikan dan mengkategorikan MRSA yang diambil dari sistem penghawa dingin yang terdapat di Fakulti Sains dan Teknologi Sumber, UNIMAS. Sampel swab diisolasikan dari sistem ventilasi penghawa dingin sentral dan diletakkan ke dalam 0.1 % air pepton. Sampel tersebut kemudiannya dibawa untuk dijalankan ujian pencairan bersiri dan seterusnya penyaduran di atas mannitol salt agar (MSA), serta diinkubasikan selama 24-36 jam dalam suhu bilik. Hanya 16.67% daripada 24 sampel dengan faktor pencairan 10^{-5} disyaki positif S. aureus, dan diteruskan untuk menjalani ujian biokimia. Namun, hanya 25% daripada sampel isolasi diuji positif bagi ujian catalase, hidrolisis gelatin, methyl red, kecuali ujian sitrat dan urease. Kesemua sampel isolasi kemudian diuji ketahanannya terhadap empat antibiotik dengan menggunakan kaedah difusi disk. Kesemua sampel isolasi dianggap tahan (0 mm zon perencatan) terhadap methicillin, penisiliin, vancomycin, dan cephalothin. Kajian ini penting bagi meningkatkan kesedaran staf-staf UNIMAS, pensyarah dan para pelajar dengan menyediakan data asaskepada pihak berwajib UNIMAS tentang status MRSA yang terdapat dalam sistem penghawa dingin di UNIMAS.

***Kata kunci**; Methicillin-resistant Staphylococcus aureus (MRSA), mannitol salt agar (MSA), methicillin, kaedah difusi disk.*

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LIST OF ABBREVIATIONS

Ammonium salts	$NH_4H_2PO_4$
Beta-lactam	β-lactam
Colony forming units	CFU
Community-acquired	CA-MRSA
Healthcare-associated	HA-MRSA
Hydrogen peroxide	H_2O_2
Luria Bertani broth.	LB broth
Mannitol Salt Agar	MSA
Mueller-Hinton Agar	MHA
Methicillin-resistant *S. aureus*	MRSA
Methicillin-sensitive *S. aureus*	MSSA
Microlitre	mm
Millilitre	ml
Millimetre	µl
Oxygen	O_2
Penicillin-binding proteins	PBPs
Peptone Buffered Saline	PBS
Potential of hydrogen	pH
Staphylococcus aureus	*S. aureus*
Vancomycin intermediate-resistant *S. aureus*	VISA
Water	H_2O

1.0 INTRODUCTION

National Institute of Allergy and Infectious Diseases (2017) reported that *Staphylococcus aureus*, which normally known as staph, was found in the 1880s and in 1940s, the infection of *Staphylococcus aureus* were successfully overcame with the aids in the discovery of antibiotic, such as penicillin. But in 1961, the first strains of *S. aureus* that possessed resistance towards methicillin was discovered, which this was the first emergence of Methicillin-resistant *Staphylococcus aureus* (MRSA). Methicillin-resistant *Staphylococcus aureus* (MRSA) is nowadays the most frequently identified antibiotic-resistant pathogen in the world, including Europe, America, North Africa, the Middle East, and also East Asia. Ippolito *et al.* (2010) stated that methicillin was first introduced in 1959 in order to treat infections produced by penicillin-resistant *S. aureus*. In 1961 it was reported from United Kingdom that *S. aureus* isolates that had obtained resistance to methicillin, and since early 1970s, the occurrence of this resistance has continuously increased (Ippolito *et al.*, 2010).

MRSA is a type of *Staphylococcus aureus* which possess resistance towards antibiotics that are called beta-lactams. Beta-lactam antibiotics are among the most frequently prescribed drugs, characterized together based on their similar structural feature, which is the beta-lactam ring (Letourneau, 2017). Examples of these antibiotics include methicillin and also other typical antibiotics, for instance oxacillin, penicillin, and also amoxicillin. Majority of MRSA infections are skin infections and assumed to be non-invasive. Letourneau (2017) further said that when a person is being infected by MRSA, but it does not cause any illness towards that individual, it is called as "colonization". According to Dani (2014), colonization defined as the presence of bacteria on a body surface without producing any disease to the individual.

Gomph (2017) explained that there are two main methods on how MRSA able to infect people. Firstly is through physical contact with infected person or a carrier (people who are not infected but are colonized with the bacteria on their body surfaces) of MRSA. The other way is for by having a physically contact MRSA from objects, for instances door handles, floors, sinks, or towels that have been touched by a MRSA-infected individual or carrier.

MRSA is hard to treat because of its resistance towards mostly of existing antibiotics. The emergence of methicillin resistance was accompanied by the development of resistance to most of the non-beta-lactam antibiotics and resulted in the reduction in options for treating infections caused by MRSA. Treatment with vancomycin, which is a glycopeptide antibiotic which always considered as a last line of defence against MRSA, has led to the emergence of vancomycin-resistant *S. aureus* (VRSA), (Rogers, 2018). In addition, Rogers (2018) added that the usage of teicoplanin, an antibiotic derived from vancomycin, also has given rise to teicoplanin-resistant MRSA strains.

MRSA causes a mild to severe infections which its infections are hardly to treat due to its resistance to several antibiotics and transmission of many virulence factors. Chew *et al.* (2018) carried out study in Malaysia to MRSA, to determine the mechanism of antibacterial activity of *Bauhinia kockiana* that arise from Peninsular Malaysia towards MRSA. Sit *et al.* (2017) reported that the prevalence rate of MRSA infection appeared to be the highest in Asia, however there is still a small number of epidemiological study carried out in Malaysia. Furthermore, there are still a poor understanding on the circumstances that mediating the ability to cause outbreaks in healthcare facilities (Boswihi & Udo, 2018).

Based on a study conducted by Shiomori *et al.* (2001), Methicillin-resistant *S. aureus* was recirculated among the patients, the air, and the inanimate environments,

especially when there was movement in the rooms. Shabha and Higgins (2011) stated that *S. aureus* is able to tolerate desiccation at a higher temperature (18°C to 37°C), and this characteristic makes it more likely to spread through ventilation and air conditioning systems.

The objectives of this study are:

1) To determine the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) from air conditioning system in Faculty of Resource Science and Technology, UNIMAS.
2) To isolate the methicillin-resistant *Staphylococcus aureus* (MRSA) from air conditioning system in Faculty of Resource Science and Technology, UNIMAS successfully.
3) To determine the presence of methicillin-resistant *Staphylococcus aureus* (MRSA) based on biochemical and antibiotic resistance testing.

Problem statement:

The prevalence and exposure towards methicillin-resistant *Staphylococcus aureus* in air conditioning system remains unclear. As UNIMAS is now entering its 25th year of establishment, it is essential to determine if the air conditioning system do not harbour pathogenic organism. This will provide basic information to the relevant authorities and staff on the risk associated with this organism.

2.0 LITERATURE REVIEW

2.1 Background of *Staphylococus aureus*

National Institute of Allergy and Infectious Diseases (2017) reported that *Staphylococcus aureus*, which normally known as staph, was found in the 1880s, where its infection normally created a skin condition such as boils, scalded-skin syndrome, and also impetigo, which are very hurting to the person infected. Furthermore, a more serious form of *S. aureus* infections are able to cause bacterial pneumonia and bacterial infection in our bloodstream, which both can cause mortality. In the past two decades, there were two clear shifts in the scope of epidemiology of *S. aureus* infection. Firstly, an increasing number of cases of health care associated with *S. aureus* infection, and the other one is that and epidemic of society having skin and soft tissue infections caused by *S. aureus* infection along with their resistance towards β-lactam antibiotics (Tong *et al.*, 2015).

2.2 Emergence of Methicillin-resistant *Staphylococcus aureus*

In 1940s, the infection of *Staphylococcus aureus* were successfully overcame with the aids in the discovery of antibiotic, such as penicillin. However, from that point onwards, due to its usage, as well as its overuse and misuse of antibiotics, the bacteria have been able to gain resistance to those antibiotics through evolution (National Institute of Allergy and Infectious Diseases, 2017). Therefore, methicillin, which is a form of penicillin was produced and used to counter back the infection of penicillin-resistant *S. aureus*. But in 1961, the first strains of *S. aureus* that possessed resistance towards methicillin was discovered, which this was the first emergence of Methicillin-resistant *Staphylococcus aureus* (MRSA). MRSA have been known as an essential human pathogens which caused from nosocomial infections globally (Montazeri *et al.*, 2014).

2.3 Community-acquired (CA-MRSA) and healthcare-associated (HA-MRSA)

According to Amin and Batts (2018), there are two types of *S. aureus* which are methicillin-sensitive *S aureus* (MSSA) and methicillin-resistant *S aureus* (MRSA). Under MRSA strains, there are another 2 types which are categorised as community-acquired (CA-MRSA) and healthcare-associated (HA-MRSA). Infections obtained by persons who have not been hospitalized recently (within the past year) or had a medical procedure (for instance dialysis, surgery, or catheter) are defined as CA-MRSA infections (Amin & Batts, 2018). Abigail (2008) categorised HA-MRSA as the infection in a person if the patient was in a high-risk healthcare site not long before diagnosis, or has been admitted in a facility for more than 72 hours before the infection starts to appear.

2.4 Recent studies of Methicillin-resistant *Staphylococcus aureus* in Malaysia

Soo (2016) reported that over 50% of *Staphylococcus aureus* infection are caused by Methicillin-resistant *S. aureus* (MRSA). MRSA is any type of *S. aureus* that gains resistance towards penicillins such as methicillin, oxacillin and flucloxacillin, and Malaysia alone recorded a high percentage of incidence occurred which were 32% (Soo, 2016). Over 50% of incidence were highly widespread and were reported in Asia, Malta, North and South America, and in Malaysia, the widespread of MRSA was estimated from 17% in 1986 to 44.1% in 2007 (Sit *et al.*, 2017). They further added in the journal that the prevalence rate of MRSA infection appeared to be the highest in Asia, however there is still a small number of epidemiological study carried out in Malaysia.

2.5 Disc diffusion method

Antibiotic resistance testing can be applied for discovery of drug, epidemiology, and also prognosis of medicinal result. Various of laboratory technique can be applied to assess or screen the in vitro antimicrobial action of an extract or a pure compound. One of the most popular technique is the disc-diffusion method. Balouiri *et al.* (2015) said that in this common method, agar plates are introduced with a standardized inoculum of the test microorganism. Next, 6 mm in diameter of filter paper discs that composed of desired compound at desired concentration are placed onto the surface of the agar plate.

The plate are then incubated under appropriate conditions. Normally, the antimicrobial compound diffuses into the agar and prevents development and growth of the test microorganism and then the diameters of zone of inhibition will be measured. The disk diffusion method is conducted by using Mueller-Hinton Agar (MHA), which is the most appropriate medium for regular antibiotic resistance tests due to its good reproducibility, low in sulfonamide, trimethoprim, and tetracycline inhibitors, and gives beneficial growth of most bacterial pathogens (Tendencia, 2004).

2.6 Air conditioning system

Air conditioning systems is used as a temperature cooling appliance in adjusting air temperature at home, offices, and other buildings for many purposes. It comprised with centralized component that gives an ambiance with manageable temperature, humidity, as well as purity whenever desired, and regardless of the climate state. However, these kinds of artificial environments give advantage for the growth of fungi, bacteria, protozoan and mites, which likely will cause health effect to the users, either through hypersensitivity or by infections. Anas *et al.* (2016) reported that microorganisms such as *Legionella pneumophilla*, *Streptococcus pneumonia* and *Bacillus spp* exist in air conditioner filters. In addition, study conducted by Modebelu and Modebelu (2013) had proved that air conditioner has the highest propensity to harbour and spread microorganisms if compared with other ventilation equipment that are used to provide a better air quality.

2.7 Airborne bacteria

Yassin and Almouqatea (2010) reported that when individuals get exposed to bio-aerosols that consist of airborne bacteria as well as their secondary metabolites, it can lead to respiratory problems and other health complications, for instance infections, hypersensitivity pneumonitis and also toxic response. In the air surrounding, moulds and microorganisms will become airborne and thus ubiquitous, because they able to pass through indoor areas by passive ventilation or via ventilation systems (Yassin and Almouqatea, 2010). Study conducted by Rajasekar and Balasubramaniam (2011) revealed that the dominant airborne microorganisms genera were *Staphylococcus, Pseudomonas, Alcaligens,* and *Corynebacterium.*

2.8.1 Catalase test

Catalase is the enzyme that breaks hydrogen peroxide (H_2O_2) into H_2O and O_2. Hydrogen peroxide is always used as a disinfectant in wounds, and the immediate bubbling formation observed is due to the production of O_2 gas. H_2O_2 is produced by some bacteria as an oxidative end product of the aerobic breakdown of sugars, and if it highly toxic to bacteria if accumulated and can lead to apoptosis (Public Health England, 2014). Since H_2O_2 is unstable, the positive and negative control should be carried out continuously.

2.8.2 Methyl red test

The Methyl-Red test tests for the potential of the bacteria to carry out mixed-acid fermentation. After incubation, the pH indicator Methyl Red is added to the broth. Methyl Red is red at pH below 4.4 (indicate positive result) and yellow at pH above 6.0. An orange colour shows an intermediate pH and would be assumed as a negative result (Hemraj, Diksha, and Avneet, 2013).

2.8.3 Citrate test

Citrate utilisation test is performed to test the capability of an organism to use sodium citrate as a sole source of carbon and ammonium salt as a sole source of nitrogen (Pradhan, 2013). Bacteria that able to grow in the medium will turn the medium alkaline. This is shown by the change of colour of bromothymol blue indicator from green to blue. Simmons Citrate agar is inoculated on the slant by touching a colony that is 18-24 hrs old with a wire loop. Inoculation from the broth culture should not be conducted since the inoculum will be too heavy. Then, it will be incubated at 35°C-37°C for up to 7 days, and the blue colouration of the media is observed.

2.8.4 Urease test

According to Public Health England (2014), the urease test is performed to determine the potential of an organism to break urea, through the formation of the enzyme urease. Two units of ammonia are produced with resulting alkalinity in the presence of the enzyme, and the increased pH is detected by a pH indicator. Christensen's urea medium consists of the pH indicator phenol red which is yellow if under acid conditions (pH 6.8) and changes into rose pink in alkaline conditions (pH 8.4). The slope is inoculated heavily over the all surface and stabbed with loop wire, then it will be incubated at 35°C-37°C in an incubator. After that, the slopes will be observed after 4 hours and after overnight incubation.

2.8.5 Gelatin hydrolysis

In 1926, Frazier explained the main method to screen the ability of microorganisms to liquefy gelatin (Cruz and Torres, 2012). Thirst (1957) stated that gelatinase activity in bacteria is determined by methods which normally need the progeny of a small inoculum to liquefy relatively big amounts of substrate at a temperature unsuitable to the development of the organism. It determined the capability of bacteria to form gelatinases. It also able to differentiate the gelatinase-positive, pathogenic *Staphylococcus aureus* from the gelatinase-negative, and also non-pathogenic *Staphylococcus epidermidis*.

2.9.1 Antibiotic resistance

Physicians were finally forced to discard their belief that, given the vast array of effective antimicrobial agents, virtually all bacterial infections were treatable in the early 1970s (Lowy, 2003). Their confidence was shaken by the appearance of resistance to many antibiotics among pathogens for examples *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis*. Foster (2017) stated that resistance can either formed by horizontal transmission of resistance determinants encoded by mobile genetic elements via plasmids, transposons, and the staphylococcal cassette chromosome, or by mutations in chromosomal genes.

2.9.2 Methicillin

In 1961, emergence of methicillin as the first of the semisynthetic penicillinase-resistant penicillin was speedily followed by reports of methicillin-resistant isolates (Lowy, 2003). Based on Stapleton and Taylor (2002), methicillin, a β-lactam antibiotic, reacts by inhibiting penicillin-binding proteins (PBPs) that are included in the formation of peptidoglycan, an important mesh-like polymer that surrounds the cell. They further explained that *Staphylococcus aureus* can become resistant to methicillin and other β-lactam antibiotics from the expression of a foreign PBP, PBP2a, that is resistant to the reaction of methicillin however still can functioning as the host PBPs (Stapleton and Taylor, 2002). Methicillin-resistant *S. aureus* isolates are always resistant to other groups of antibiotics, via variety of mechanisms which making treatment choices narrow, and this has resulted to the seek for new compounds active towards these strains.

2.9.3 Penicillin

Penicillin was introduced in 1928 but it was not utilized in clinical practice prior to 1941 (Cheng *et al.*, 2016). Widespread use of penicillin was followed by the formation of penicillin resistance among *Staphylococcus aureus* isolates within four years. Cheng *et al.* (2016) further explained that the widespread penicillin usage quickly led to the formation of penicillin resistance in *S. aureus* and new data proposed that penicillin susceptibility may be in a period of renaissance. (Cheng *et al.*, 2016). According to Hagstrand, Skovby, and Pahlman (2017), penicillin is now seldom used in the clinical practices of *S. aureus* infections due to widespread resistance and a worry about the exactness of existing techniques for penicillin susceptibility testing.

2.9.4 Cephalothin

Cephalothin is a first generation cephalosporin antibiotic and remains to be widely used (Choudhary, 2017). Cephalosporins are beta-lactam antimicrobials that have same mechanisms of action and a similar structure with penicillins. Harrison and Bratcher (2008) explained that penicillins and cephalosporins possesses the same four-member "core" beta-lactam ring, however cephalosporins have an extra atom in the side ring. Mechanism of action of cephalosporins consist of a rigid bacterial cell walls that can be assumed as a series of repeating interlinking units reminiscent of floor tiles (Harrison and Bratcher, 2008). During replication, a bacterium discards "tiles" circumferentially to let cell division through a pinching-like action, while rapidly substituting new "tiles" at the ends of what have become two bacteria. This process needs enzymes to interlink replacement tiles. Such enzymes are the aims of beta-lactam antibiotics and are known as penicillin-binding proteins (PBPs). Antibiotic process requires binding to PBPs, averting them from closing the vulnerable ends on dividing bacteria and resulting the natural intrabacterial hyperosmotic pressure to break down the bacteria. Therefore, beta-lactam antibiotics are bactericidal.

2.9.5 Vancomycin

Methicillin-resistant *Staphylococcus aureus* probably sensitive to some other antibiotics, such as clindamycin, macrolides, tetracyclines, trimethoprim-sulfamethoxazole and quinolones, or resistant to all antibiotics except vancomycin (Hakim *et al.*, 2007). Hakim *et al.* (2007) explained that vancomycin stayed as the only predictable active antibiotic towards all strains of *S. aureus*, and especially methicillin-resistance *S. aureus*. Vancomycin, a glycopeptide antibiotic that obstructs cell wall biosynthesis, stays as a best choice of drug for treatment of serious MRSA infections. According to McGuinness *et al.* (2017), *S. aureus* strains showing increased resistance towards vancomycin, which called vancomycin intermediate-resistant *S. aureus* (VISA) were first known in the 1990s. The molecular basis of resistance in VISA is polygenic and involves stepwise mutations in genes encoding molecules predominantly included in cell envelope biosynthesis (McGuinness *et al.*, 2017). *S. aureus* isolates with fully resistance towards vancomycin are called as vancomycin-resistant *S. aureus* (VRSA), and they were first reported in the U.S. in year 2002.

3.0 MATERIALS AND METHODS

3.1 Swab samples collection

A total of 24 swab samples were collected from 12 lecturers' offices, with each of the offices were chosen as the representative of the main office based on its location. The offices are located in Faculty of Resource Science and Technology, UNIMAS. The swab samples were collected from the evaporator of the central air conditioning system. The cotton bud was dipped into a sterile water prior to swabbing. After the swab samples collected, it was inserted into the Bijou bottle, till the bottom of the medium of 3 ml of 0.1 % peptone water, and were kept in icebox. The swab samples were then transported to the Microbiology laboratory as soon as possible, and were proceeded to serial dilution. Table 3.1 shows the detail location of the samplings taken. Two offices were chosen as the representative at each location, and all of the swab samples were collected from central air conditioning system.

Table 3.1: Detail location of the sampling

Location	Lecturer's offices	Types of air conditioning system	Samples
G1	A	Central	S1D1
		Central	S1D2
	B	Central	S2D1
		Central	S2D2
G2	C	Central	S3D1
		Central	S3D2
	D	Central	S4D1
		Central	S4D2
G3	E	Central	S5D1
		Central	S5D2
	F	Central	S6D1
		Central	S6D2
G4	G	Central	S7D1
		Central	S7D2
	H	Central	S8D1
		Central	S8D2
G5	I	Central	S9D1
		Central	S9D2
	J	Central	S10D1
		Central	S10D2
G6	K	Central	S11D1
		Central	S11D2
	L	Central	S12D1
		Central	S12D2

3.2 Determination of colony forming unit

The swab samples in the Bijou bottles were first homogenised for 30 seconds by using the vortex mixture. After that, 1 ml of the sample was then transferred into a tube that contained 9 ml of Peptone Buffered Saline (PBS) buffer. This was considered as 10^{-1} dilution factor. Then, 1 ml of the previous tube was transferred into another tube that contained 9 ml of PBS buffer. The dilutions were proceeded until 10^{-5} dilution factor. 100 µl of samples from the tubes of 10^{-4} and 10^{-5} were each dropped onto Mannitol Salt Agar (MSA), with duplicate for each dilution. The plates were then incubated for 24-36 hours. Then, the readings of colony forming units (CFU) were calculated and recorded. The following equation was used to compute the CFU per ml.

$$\text{CFU per ml} = \frac{\text{Average number of colonies for a dilution x dilution factor}}{\text{Volume of sample taken (ml)}}$$

3.3 Preliminary screening for identification of suspected *Staphylococcus aureus*

After done the serial dilutions, the samples were then proceeded to gram staining followed by biochemical tests (El-Hadedy and El-Nour, 2012). The colonies that showed the characteristics of *Staphylococcus aureus*, which were gram-positive cocci in clusters were identified. Next, the pre-identified suspected *S. aureus* were then undergo several biochemical testing such as catalase test, gelatin liquefaction, methyl red, citrate, and also urease tests for further confirmation. For catalase test, 3% of H_2O_2 was dropped onto a slide contained a colony of overnight culture of sample, and the immediate formation of bubbles were observed. Then for gelatin liquefaction, the tubes that contained 24 hours colony and gelatin were checked for liquefaction, and as for methyl red test, the development of red colour after addition of methyl red reagent were considered as positive result. As for citrate tests, the changed of colour of Simmon's Citrate agar from green to blue were indicated

positive results. Finally for urease test, changes of colour of Christensen's Urea agar from light orange to pink showed a positive result.

3.4 Disc diffusion method

All of the samples from biochemical tests were then moved on to undergo antibiotic resistance test (Gandara *et al.*, 2006). The samples were taken from a tube contained overnight culture in 5 ml of Luria Bertani (LB) broth. Next, a disc diffusion method was applied for the sample. A microbial suspension of 0.5 McFarland was used as reference for turbidity. The surface culture was conducted by using swab on Mueller-Hinton agar plate. The disks containing different antibiotics which were methicillin, penicillin, cephalotin, and ampicillin were put on the surface of the culture with a suitable distance from each other and from the edge of the plate. The plates were incubated for 48 hours before the results of antibiotic resistance were taken. After 48 hours, the diameter of clear zone of inhibition were measured and recorded (Gandara *et al.*, 2006). The zone diameter of inhibition was categorised as resistant when it was less than 7 mm, relatively resistant when it was 7-9 mm, relatively sensitive when it was 10-12 mm, and sensitive when the diameter was more than 12 mm (Koohsari *et al.*, 2015).

4.0 RESULTS

The suspected *Staphylococus aureus* appeared whitish in colony on mannitol salt agar (MSA). From all of 24 swab samples collected from the ventilation of central air conditioning system, only four of them were resulted in gram-positive bacteria and have shape of cocci in clusters. Serial dilution was carried out and only dilution factor of 10^{-4} and 10^{-5} were calculated, since the dilution factor above them were too numerous to count. Meanwhile for the other samples resulted in gram-negative bacteria with short rod in shape. The result are summarized in Table 4.1, that shows the CFU unit per ml, the morphology of the colonies which appeared on MSA agar, gram stain (positive or negative), and also which samples were suspected as *S. aureus*. In the other hand, Figure 4.1 shows the appearance of whitish colony of suspected *S. aureus* on MSA agar. The suspected colony of *S. aureus* appeared in big and round whitish colony. Figure 4.2 shows the morphology of suspected colonies of *S. aureus* under light microscope. The suspected colonies of *S. aureus* appeared to be in grape-like clusters and gram positive (purple in colour) when viewed under microscope.

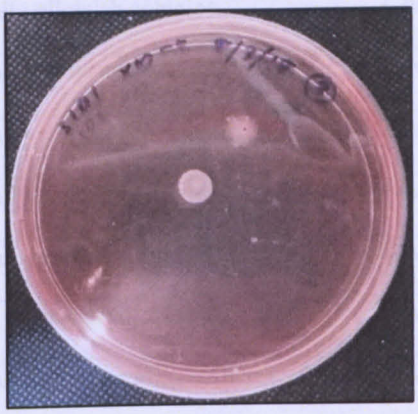

Figure 4.1: Whitish colony of suspected *S. aureus* on MSA agar.

Figure 4.2: Morphology of suspected *S. aureus* with gram-positive (purple in colour) and have shape of cocci in clusters (40x10 magnification).

Table 4.1: CFU unit per ml, colony appearance on mannitol salt agar, gram stain, and the suspected *S. aureus*.

Sample	Dilution factor $x10^{-5}$ (cfu/ml)	Colony appearance on Mannitol Salt Agar	Gram stain	Suspected *S. aureus*
S1D1	$3.5x10^{6}$	Small white colonies	(+)ve	Yes
S1D2	0	Small yellowish colonies	(-)ve	No
S2D1	$5.0x10^{5}$	Small yellow and white colonies	(+)ve	Yes
S2D2	$7.3x10^{7}$	Small yellowish colonies	(-)ve	No
S3D1	$1.8x10^{7}$	Small yellowish colonies	(-)ve	No
S3D2	$4.45x10^{7}$	Small yellowish colonies	(-)ve	No
S4D1	$4.65x10^{7}$	Big yellowish colonies	(-)ve	No
S4D2	$6.0x10^{6}$	Small and big reddish colonies	(-)ve	No
S5D1	0	Small reddish colonies	(-)ve	No
S5D2	$1.445x10^{8}$	Small yellowish colonies	(-)ve	No
S6D1	$9.0x10^{7}$	Small and big yellowish colonies	(-)ve	No
S6D2	$5.0x10^{6}$	Big yellowish colonies	(-)ve	No
S7D1	$3.0x10^{6}$	Small white colonies, and big yellowish colonies	(+)ve	Yes
S7D2	$2.25x10^{7}$	Big yellowish colonies	(-)ve	No
S8D1	$6.3x10^{7}$	Small yellowish colonies	(-)ve	No
S8D2	$8.5x10^{6}$	Small white colonies	(+)ve	Yes
S9D1	$1.03x10^{8}$	Small yellowish colonies	(-)ve	No
S9D2	$4.95x10^{7}$	Small yellowish colonies	(-)ve	No
S10D1	$1.7x10^{7}$	Big yellowish colonies	(-)ve	No
S10D2	$1.5x10^{7}$	Big yellowish colonies	(-)ve	No
S11D1	TNTC	Small and big yellowish colonies	(-)ve	No
S11D2	$2.385x10^{8}$	Small and big yellowish colonies	(-)ve	No
S12D1	$1.79x10^{8}$	Small yellowish colonies	(-)ve	No
S12D2	$3.4x10^{7}$	Big yellowish and whitish colonies	(-)ve	No

Out of 24 samples, only four (25%) of them were tested positive for the suspected *S. aureus*. Therefore, all four of the isolates were then proceeded for the biochemical tests. The biochemical tests conducted were catalase test, gelatin liquefaction test, methyl red test, citrate test, and also urease test. Out of those four isolates, only one of them were mostly tested positive for all the biochemical tests conducted, except for citrate test and urease test, where they showed negative results. For the other isolates, all were tested negative for catalase test, gelatin liquefaction test, citrate test, and urease test. However, all of the isolates gave positive result for methyl red test. Figure 4.3 shows the positive and negative result of catalase test which the positive result gave an immediate bubble formation after the isolates being dropped with 3% H_2O_2, and also negative results which showed no reaction after the isolates being dropped with 3% H_2O_2. Figure 4.4 shows results of gelatin liquefaction, which positive result showed the liquefaction of gelatin after being stabbed with the isolate and incubated in suitable condition, and also the negative result which showed the solidification of galatin after the same treatment applied. Figure 4.5 shows the results of methyl red test which the positive result showed colour changes of the MRVP broth from yellow to red after being dropped with methyl red reagent and negative result which showed no colour changes after being dropped with the same reagent. The results are summarized as in Table 4.2 which shows the results of each biochemical tests which are catalase test, gelatin hydrolysis, citrate test, and also urease test for each isolates (positive or negative).

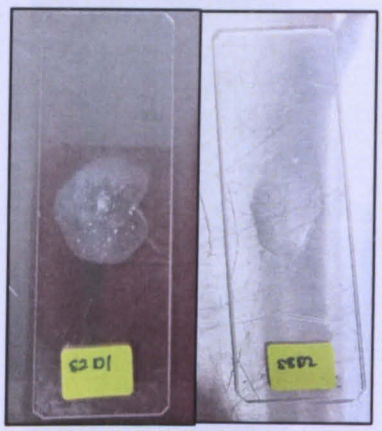

Figure 4.3: Reaction after the isolate was dropped with 3% H_2O_2. Immediate bubble formation (positive result on left side) and no reaction (negative result on right side).

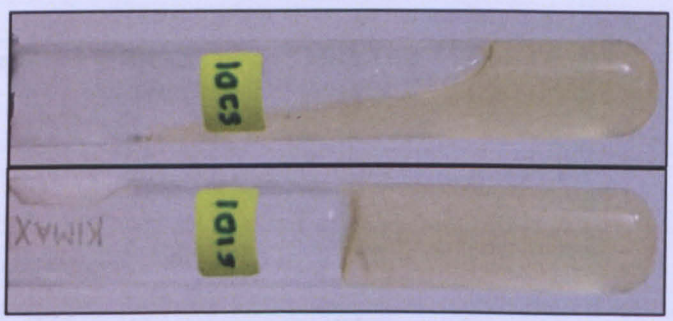

Figure 4.4: Gelatin liquefaction (positive result for picture at above) and gelatin solidified (negative result for picture at bottom).

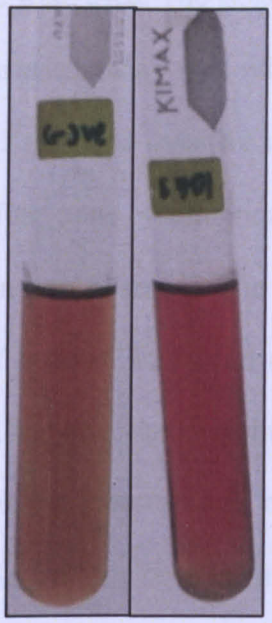

Figure 4.5: No changes in colour of the MRVP broth (negative result on left side) and changes in colour from yellow to red (positive result, after being dropped by methyl red reagent on right side).

Table 4.2: Results of catalase test, gelatin hydrolysis, methyl red test, citrate test, and also urease test for the samples.

Sample	**Biochemical test**				
	Catalase	Gelatin liquefaction	Methyl red	Citrate	Urease
S1D1	(-)ve	(-)ve	(+)ve	(-)ve	(-)ve
S2D1	(+)ve	(+)ve	(+)ve	(-)ve	(-)ve
S7D1	(-)ve	(-)ve	(+)ve	(-)ve	(-)ve
S8D2	(-)ve	(-)ve	(+)ve	(-)ve	(-)ve

For the antibiotic resistance test, no zone of inhibition were observed. Therefore, since 0 mm diameter of zone of inhibition were measured, 100% of the isolates were categorised as resistant towards methicillin, cephalothin, penicillin, and also vancomycin.

The zone of inhibition is a clear zone surround the paper disc, which in this test conducted, the paper disc containing antibiotic were used. The isolates will be categorised as resistant when its diameter zone of inhibition is less than 7 mm, relatively resistant when it is 7-9 mm, relatively sensitive when it is 10-12 mm, and sensitive when the diameter is more than 12 mm (Koohsari *et al.*, 2015). Therefore, zone of inhibition for all the isolates were recorded as 0 mm, since no zone of inhibition were observed. The results are summarized as in Table 4.3 which shows the diameter of zone of inhibition (mm) for antibiotic resistance of methicillin, cephalothin, penicillin, and also vancomycin towards suspected MRSA. All of the isolates gave 0 mm zone of inhibition towards all of the antibiotic tested. Figure 4.6 shows the antibiotic resistant activity on Mueller-Hinton (MH) agar. No zone of inhibition by antibiotic (methicillin, penicillin, cephalothin, and vancomycin) on *S.aureus* that have been swabbed on MH agar were observed after 48 hours of incubation period.

Table 4.3: Zone of inhibition (in diameter) for antibiotic resistance of methicillin, cephalothin, penicillin, and also vancomycin towards suspected MRSA.

Antibiotics / Isolates	Diameter of clear zone (mm)			
	Methicillin (5μg)	Cephalothin (30 μg)	Penicillin (1 μg)	Vancomycin (30 μg)
S1D1	0	0	0	0
S2D1	0	0	0	0
S7D1	0	0	0	0
S8D2	0	0	0	0

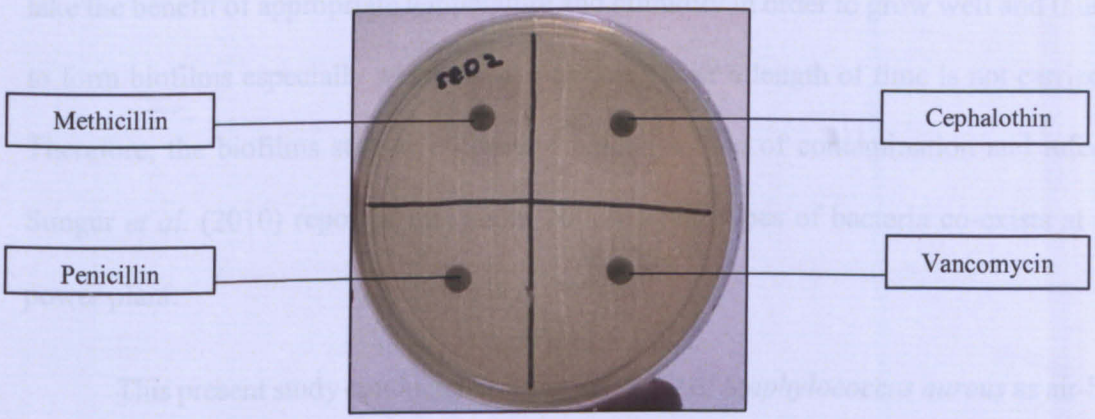

Figure 4.6: No zone of inhibition by antibiotic (methicillin, penicillin, cephalothin, and vancomycin) on *S.aureus* that have been swabbed on Mueller-Hinton agar.

5.0 DISCUSSION

The air conditioning system is one of ventilation device available for the use of controlling and enhancing the quality of air circulating in indoor environment. It gives a comfortable environment for occupants of building, rooms or vehicles when using it. However, the air conditioning system is one of bacterial habitat where pathogenic bacteria may live on it. This statement was supported by (Modebelu and Modebelu, 2013), in their statement which they stated that vary of bacterial isolates were found as significant contaminants of wide types of ventilation equipment including *Staphylococcus aureus* and *Staphylococcus epidemidis*.

Those bacteria resides in the air conditioning system by using all of the available nutrients circulating in the environmental aerosols in order to feed themselves. They also take the benefit of appropriate temperature and humidity in order to grow well and thus lead to form biofilms especially when surface cleaning over a length of time is not carried out. Therefore, the biofilms stay as a constant hidden source of contamination and infection. Sungur *et al.* (2010) reported that about 200 different types of bacteria co-exists at some power plant.

This present study conducted is as an indicator of *Staphylococcus aureus* as air-borne bacteria status in our indoor environmental air as well as their ability to withstand multiple types of antibiotic tested. Air-borne bacteria multiply and circulate in our indoor environment without us knowing their existence that will have bad impact on our health, as they will cause a poor air quality in our indoor environment. Study carried out by Modebelu and Modebelu (2013) proved that air conditioner has the highest tendency to harbour and spread microorganisms if compared with other ventilation equipment that are used to give a better air quality.

Based on the result of the study conducted, the colony that have the similar characteristics of *Staphylococcus aureus* when observed under light microscope appeared to be in whitish colony on mannitol salt agar (MSA). After being treated with gram stain and observed under light microscope with 100x magnification, the whitish colony appeared to be gram-positive coccus, and seems to be like grape-like clusters. According to Foster (1996), staphylococci are gram-positive with range 0.5-1.0 μm in diameter and usually grow in clusters, pairs and in short chains. The disposition of staphylococci and micrococci can be differentiate from streptococci since streptococci normally grow in chains (Foster, 1996).

Out of 24 swab samples taken from the ventilation of central air conditioning system, only 16.66% gave the result of gram-positive bacteria, with the same morphology of *Staphylococcus aureus* when observed under light microscope. Gandara *et al.* (2006) stated that according to the evidence showing that human activities including the motion of dry fabrics, for instance curtain movement are related with upraising concentrations of airborne bacteria. Therefore, it is can be assumed that concentrations of *S. aureus* possibly resulted higher indoors than outdoors. Since the study conducted gave only 16.66% of the samples that showed positive result of suspected *S. aureus*, it is quite surprising to find out that the prevalence of *S. aureus* in this study was quite low.

The isolates of suspected *Staphylococcus aureus* were then followed by biochemical tests which were catalase test, gelatin liquefaction test, methyl red test, citrate test, and also urease test. Only 25% of the isolates were tested positive for almost all of the biochemical tests conducted except for citrate test and urease test, meanwhile the other 75% of the isolates were only tested positive for methyl red test and negative for other biochemical tests. The isolate that resulted positive for almost biochemical test mentioned above was highly assumed as *S. aureus*. Although it showed negative result for citrate test and urease test, it

might be the result of having poor technique while conducting the test, such as streaking method.

The forest green colour of Simmon's Citrate Agar should change to blue in colour which indicate that the organism able to use citrate as carbon source and inorganic ammonium salts ($NH_4H_2PO_4$) source of nitrogen. Since *Staphylococcus aureus* can metabolize citrate, the ammonium salts will be broken down to ammonia which will elevate the alkalinity. Therefore, bromothymol blue indicator in the medium will change from green to blue above pH 7.6 as the indicator of pH changes. The test conducted may be false negative since some microorganisms are actually able to grow on citrate but do not show any colour changes. It is still assumed as positive citrate test even the colour change is absent. In addition, the reaction of this test alone is actually insufficient to determine the species they are in. Besides that, the tests which give ambiguous results should be retested.

As for urease test, which Christensen's Agar is used, the purpose is actually to identify microorganisms that are able to hydrolyse urea in order to produce ammonia and also carbon dioxide. Brink (2010) stated that the media comprise 2% urea and phenol red as the pH indicator. Therefore, since the ammonia concentration elevated, the pH will increase, thus this will result in a colour changes from yellow to bright pink. For this test, some microorganisms may hydrolyse urea instantly, and some may not. Besides that, the colonies should be obtained from pure culture when performing this test, and in order to speed up the growth and broken down processes of urea, inoculum from broth suspension should not be used (Aryal, 2015). Aryal (2016) added that the medium also should be incubated with loose caps to avoid incorrect results to occur and also that since urea is sensitive to light and able to undergo auto hydrolysis, it is recommended to store the medium at 2°C to 8°C, away from light. Any inability in complying with these precautions mentioned above, may the reason of probability having the false negative result in this study.

According to Koohsari *et al.* (2015), the zone diameter of inhibition was categorised as resistant when it was less than 7 mm, relatively resistant when it was 7-9 mm, relatively sensitive when it was 10-12 mm, and sensitive when the diameter was more than 12 mm. As for the antibiotic resistance test that had been carried out, 100% of the isolates were resulted as resistant towards all of the antibiotic tested, which are methicillin, cephalothin, penicillin and also vancomycin, since there were no inhibition zone observed (0 mm diameter of zone of inhibition). These were very significant findings since although only 16.66% of the total samples were tested positive for the suspected *Staphylococcus* aureus, 100% of them, possess a very high resistance towards all of the antibiotic tested. Therefore, based on these findings, it can be said that the samples were suspected as methicillin-resistant *S. aureus.* Reygaert (2013) said that MRSA are considered to have resistant against all penicillin and also almost all β-lactam drugs. In the report added that vancomycin had been used to control the widespread infection of MRSA, however after several years, there were emergence of strain of *S. aureus* that also had developed resistant towards vancomycin (Reygaert, 2013).

6.0 CONCLUSION

Ventilation devices especially air-conditioners, play important role in filtering and circulating quality air and providing conducive environment inside offices for example. This study discovered that air-conditioners in the offices harbouring methicillin-resistant *Staphylococcus aureus* although the prevalence was low. They may introduce and circulate periodically in indoor micro-environment in some offices, and thus affecting the occupants' health. Such appliances which is air conditioning system are therefore not 100% efficient in their present state as means to secure future air quality. MRSA is a common pathogen in many healthcare facilities and communities worldwide. It causes mild to severe infections which is sometimes hard to treat due to its resistance to multiple antibiotics and carriage of multiple virulence factors. Vancomycin was the drug of choice when the treatment using non-beta lactam antibiotics failed. However, in recent years the emergence of vancomycin-resistant *S. aureus* (VRSA) had made it difficult to treat infections caused by MRSA. Therefore, new antibiotics have been developed and some have shown good antimicrobial potency against MRSA strains. Therefore, it is better for us to prevent from being infected since prevention is always better than cure. In such a way, it will help to decrease the cost of healthcare and also mortality rate.

7.0 RECOMMENDATIONS

1. There should be monthly cleaning of air-conditioners' ventilation system and their other accessible components.

2. There should be quarterly or biannual microbiological examination of air-conditioners and surfaces of other ventilation devices for pathogens or potential microorganisms which are responsible for respiratory health significance.

3. Air-conditioners should be positioned in such a way that strong air current coming from it, shall not gain direct entry into the inner nostrils of users.

4. Cleaning campaign should be done by UNIMAS authorities, along with students and lecturers participation, in order to increase their awareness on the significance of healthy environment.

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9.0 APPENDICES

Figure 9.1: Collection of swab samples in Bijou bottles.

Figure 9.2: Swab samples put into Bijou bottle contained 0.1% peptone water.

Figure 9.3: Collection of swab samples at lecturer's office located at FRST, UNIMAS.